EMBRYOTOXIC AND TERATOGENIC EFFECTS OF SMOKELESS TOBACCO IN SWISS ALBINO MICE.

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INTRODUCTION

Some babies enter the world suffering from physical, mental or metabolic handicaps due to embryotoxicity which results from exposure to certain toxins like some drugs, solvents, heavy metals, pesticides, anesthetic gases & organic compounds and study of toxic responses in offspring to environment or drug following exposure of mother at any time from conception to birth.

Tobacco is one of the major causes for embyotoxicity. It is estimated that about 30% of all women of childbearing years smoke and 25% of all women continues on smoking during pregnancy (Brent and Beckman, 1990). As smoking is supposed to be dangerous for pregnancy and its adverse effects are well known, therefore females prefer to switch over to the use of smokeless tobacco during the pregnancy. Smokeless tobacco is used in unburnt form and is of various types such as pan masala and gutkha. The smokeless tobacco habit is difficult to break, therefore, public health efforts have been largely aimed at prevention.

About 35–40% of tobacco consumption in India is in smokeless forms, mostly of the species *Nicotiana rustica,* while most widely used smoking tobacco is *N. tabacum.*In some parts of India, such as the states of Bihar and Maharashtra, smokeless tobacco use is more common than smoking tobacco.

Awareness of the hazards of smokeless tobacco use is very low in rural populations. On the other hand, many believe tobacco, smoked or smokeless, has medicinal value for curing or Palliating common discomforts such as toothache, headache, and stomach ache.

Table 1- Number of persons reporting consumption of tobacco in various forms per 1000 persons by sex and age group (cited from NSSO 50th round 1993-94)

Form of tobacco consumption	Age group											
	10-14		15-24		25-44		45-59		60+		all ages	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
					Rural							
Smoking	5	1	120	7	434	25	498	46	398	48	293	23
Chewing zarda, dokta etc.	4	3	66	16	205	59	259	103	264	107	151	53
Snuff	0	0	4	3	7	7	12	16	13	19	7	7
Burnt tobacco powder/paste	4	5	18	20	44	39	60	55	68	52	35	33
Consumption of tobacco in any form	13	9	191	46	613	122	723	204	650	212	430	109
					Urban							
Smoking	1	0	55	2	305	5	381	18	266	18	202	66
Chewing zarda, dokta etc.	1	0	31	4	114	26	141	66	133	72	80	26
Snuff	0	0	1	1	3	3	9	8	14	19	4	4
Burnt tobacco powder/paste	2	1	6	6	19	14	28	29	25	28	15	13
Consumption of tobacco in any form	4	2	87	12	407	45	509	114	395	130	277	47

The above table presents the distribution of tobacco use by form of tobacco consumption.

REVIEW OF LITERATURE

Adverse outcomes from maternal cigarette smoking during pregnancy is well documented. It includes intrauterine growth retardation, preterm delivery, prenatal mortality, and spontaneous abortions etc.

It has been reported by Brent and Beckman, 1990 that 30% of women of child bearing age smokes, and 25% of women continues to smoke during pregnancy; and not just smoking tobacco, but similar outcomes are also shown from smokeless tobacco as well. Studies from India have shown a nearly threefold increase in stillbirths and 100–400 gm decrease in birth weight, in offspring of women who applied or chewed tobacco during pregnancy (Krishna, 1978) The studies of Beyer *et.al*.,2001 and Krishnamurthy *et.al*.,1997 have shown that 124 odds ratios varying from 2 to 3 have been found for low birth weight in infants born to mothers using smokeless tobacco.

Cigarette smoking (Nicotine) by pregnant women can also adversely affect prenatal development, birth and postnatal development. Mathews, Menaker and Doman (2003) observed that fetal and neonatal deaths are higher among smoking mothers. Fried and Watikinson, (1990) in a study found out that exposure to cigarette smoking was related to poorer language and cognitive skills at 4 years of age.Thapar *et.al.,(*2003) in a study revealed a link between maternal smoking during pregnancy and increased incidence of attention deficit, hyperactivity disorder in almost 3,000 children of 5 to 16 years of age. Klesges *et.al.,(*2001) designed intervention programmes to help pregnant women stop smoking.

Cigarette also has active ingredients that are harmful to the unborn. Lambers and Clarke (1996) observed that Nicotine which is an active ingredient of tobacco is introduced into the blood stream either by smoking, snuffing or chewing. Further processing of tobacco is displayed in different brands of cigarette, all with devastating effect on the unborn.

It is also observed that smoking and alcohol can negatively affect the mechanism of sperm life span; whether the man smokes or inhales the smoke from the smokers.

According to Kumar (2008), chewing mixtures (smokeless tobacco) are likely to be carcinogenic as tobacco and areca nut have carcinogenic addictive potential. They not only lead to cancer but may also affect other organs of body and leads to dependence on them. To prevent these hazards more research is needed so that early changes which could be reversible can be found out and also intervention measures through education to desist people in indulging in such habits are also needed.

Kumar (2008) also studied that Pan masala reduced testis weight in mice and enhanced the frequency of morphological abnormalities in mouse sperm. Areca-nut extracts were embryotoxic when given to pregnant mice during early gestation, and teratogenic when administered into the yolk sac of chick embryos. Extracts of Piper betel stalks disturbed the estrous cycle in female rats and reduced fertility in males. In mice, arecoline was genotoxic to early spermatids and it increased the frequency of abnormal sperm.

Chronic exposure to pan masala impair liver function in rats and germinal cells in mouse by inducing sperm-head abnormilities .(Mukherjee *et. al*., 1991)(Sharma *et. al*., 1992)

Pure inbred Swiss mice of both sexes were exposed to pan masala (2% concentration in feed) by Nigam *et.al.(*2001). After 56 weeks of exposure to sada pan masala, 4 out of 12 animals showed tumors. Whereas in group having tobacco as one of the constituents, 4 out of 12 showed tumors.

Study done by Ramchandani *et.al*. (1999) revealed that the body weight, survival rate of mice fed on pan masala were lower than that of controls and lifetime feeding of pan masala induced various diseases related to liver, stomach, prostrate and sebaceous glands and many other carcinogenic changes too. After the analysis of all the tumor induction data, it was shown that lung is the major target tissue for carcinogenic action of pan masala.

Plasma nicotine levels comparable to those of an average smokeless tobacco user, smokeless tobacco produces weight reduction, delayed ossification and increase in hemorrhages and fetolethality an the CD-1 mouse fetus. (Paulson *et.al*.,1988)

According to Sinha and Rao (1985a), aqueous extract of dry as well as raw/wet varieties of Betel nut were reported to be fetotoxic in Swiss albino mice leading to death, enhanced resorption and reduced weight of fetuses.

Several adverse effects on exposure to Arecoline hydrobromide (an alkaloid found in betel nut) has been well documented in different studies. These include chick embryo mortality, fetuses retarded development, reduced body size, scanty fathering, club foot.Developmental retardation of Zebra fish embryo due to cytotoxic effect by depletion of intracellular thiols (Chang *et. al*., 2001), abnormality in sperm head shape and unscheduled DNA synthesis (UDS) in spermatid stage of Swiss albino mice (Sinha and Rao, 1985b).Arecoline Induced micronuclei formation in fetal mouse blood after transplacental exposure to Betel nut is also reported (Sinha and Rao, 1985c)

Chaudhary K.,(1999) observed that the betel leaf may provide partial protection against the carcinogenic effect of tobacco, areca nut and lime mixture, catechu may be anti-carcinogenic. However, the presence of both these substances in betel quid-containing tobacco is not enough to negate the carcinogenic effect of areca nut, tobacco and lime. If the betel leaf is removed from betel quid-containing tobacco, catechu alone is not enough to negate the carcinogenic effect of the areca nut, tobacco and lime mixture.

OBJECTIVES OF THE STUDY

To investigate the possible Embryotoxic and Teratogenic effects of smokeless tobacco in Swiss Albino Mice.

To find out the effects of test materials on reproductive organs and their functions .If any.

To assess the prenatal and postnatal effects of the test materials.

MATERIALS AND METHOD

TEST MATERIALS-

Pan masala- Pan masala is a commercial preparation containing areca nut, slaked lime,catechu and condiments, with or without powdered tobacco.
Various branded products are available: Manikchand, Mahak, Rajnigndhha,Vimal, Crane,Rajdarbar, Kuber, Yamu, Badshah, Tulsi, Rahat, Betel quid King, Jubilee etc.

In present study Rajnigndha will be taken as a test material.(Sample A)

Gutkha-Gutkha is a dry, relatively non-perishable, commercial preparation containing areca nut,slaked lime, catechu and condiments and powdered tobacco (tobacco waste).The same mixture without tobacco is called pan masala.

Some common brand names are Manikchand, Moolchand, Tulsi, Shimla, Sikandar, Parag, Sir,Shikhar, Goa, Sikandar etc.
'Dilbagh 'select as a test material.(Sample B)

Preparation of extract-The sample is grounded and kept at 4°C until further use. The sample powder then will be dissolved in distilled water. Sample will be centrifuged for 20 min. The extract will be filtered. The dark brown filtrate will be given orally administration.

Maintenance of animals-Both sexes of Swiss Albino mice 6-8 weeks old will be maintained in standard condition: 23±2°C.Relative Humidity: 55±10%.12:12 h LD Cycle and allowed free access to food and water. They will be housed in separate polypropylene cages containing sterile paddy husk as bedding material.
The mice will be monitored for change in body weight and food consumption during the experiment.

METHOD-

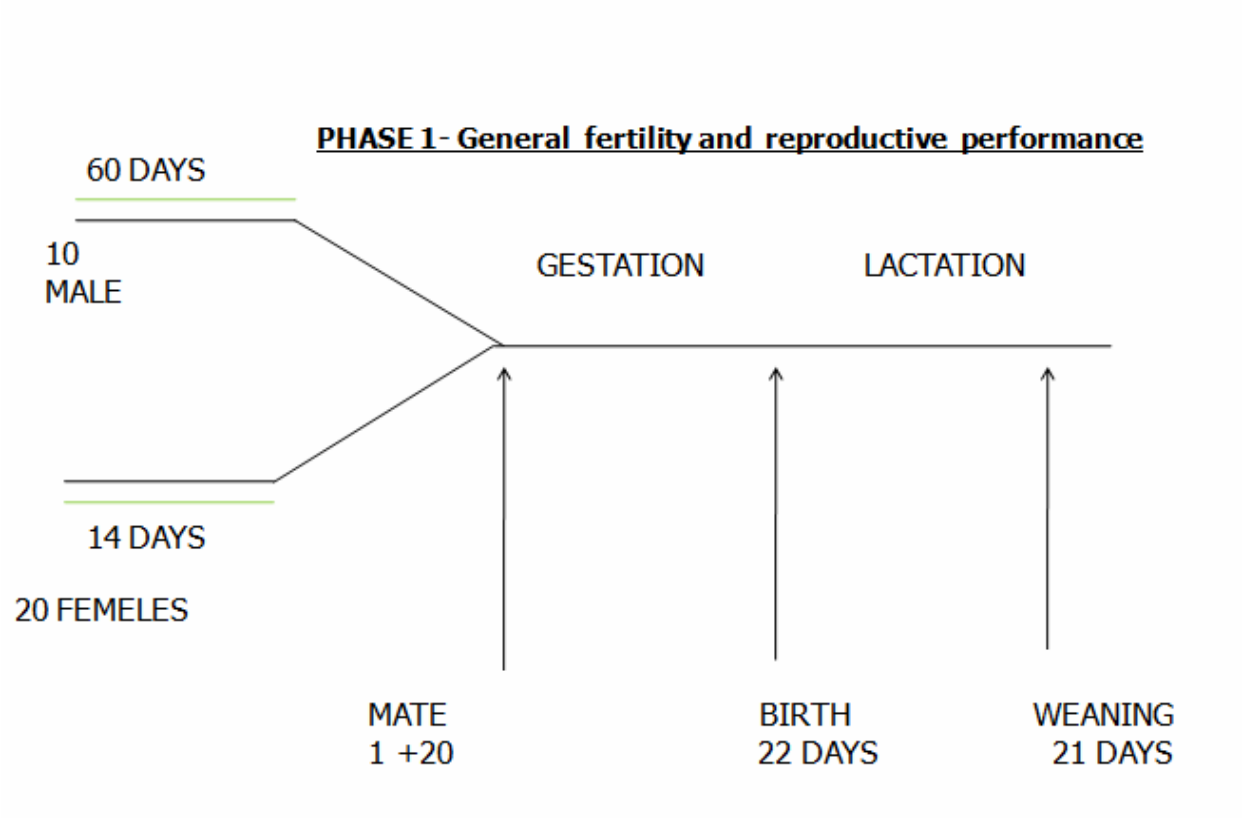
-To elucidate the teratogenic effects of smokeless tobacco on Swiss albino mice, the experiments will be conducted in 3 phases-

1-General fertility and reproductive performance

2-Teratological study

3-Perinatal/postnatal study

General fertility and reproductive performance-These test is conducted to assess the effects of a compound on gonadal function, estrous cycles, mating behavior ,conception rates and early stages of development.



Male rodents per test group will be treated for 60 days because the spermatogenesis cycle of male mice is of duration 64 days.

The temporal course of spermatogenesis

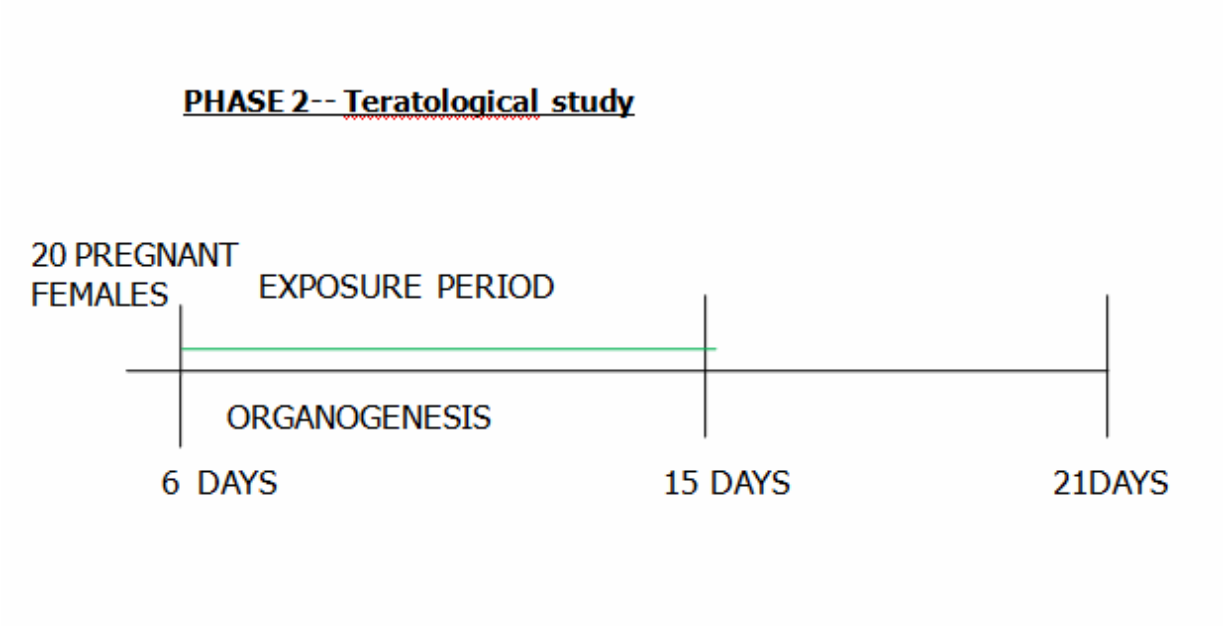
The approximate **64 day** cycle of the spermatogenesis can be subdivided into four phases that last differing lengths of time:

Mitosis of the spermatogonia	16 days	Up to the primary spermatocytes
First meiosis	24 days	For the division of the primary spermatocytes to form **secondary spermatocytes**
Second meiosis	A few hours	For engendering the **spermatids**
Spermiogenesis	24 days	Up to the completed **sperm cells**
Total	**~64 days**	

In this experiment 10 male rodents per test group will treated for 60 days and at least 20 females will expose for 14days to cover the spermatogenesis and ovulation respectively .Treated animals will be allowed to mate, inseminated females will be continued with the oral administration throughout pregnancy and lactation.

At mid pregnancy ,half of the females per test group will be sacrificed and uterine contents will examined for pre-implantation and post- implantation embryolethality.

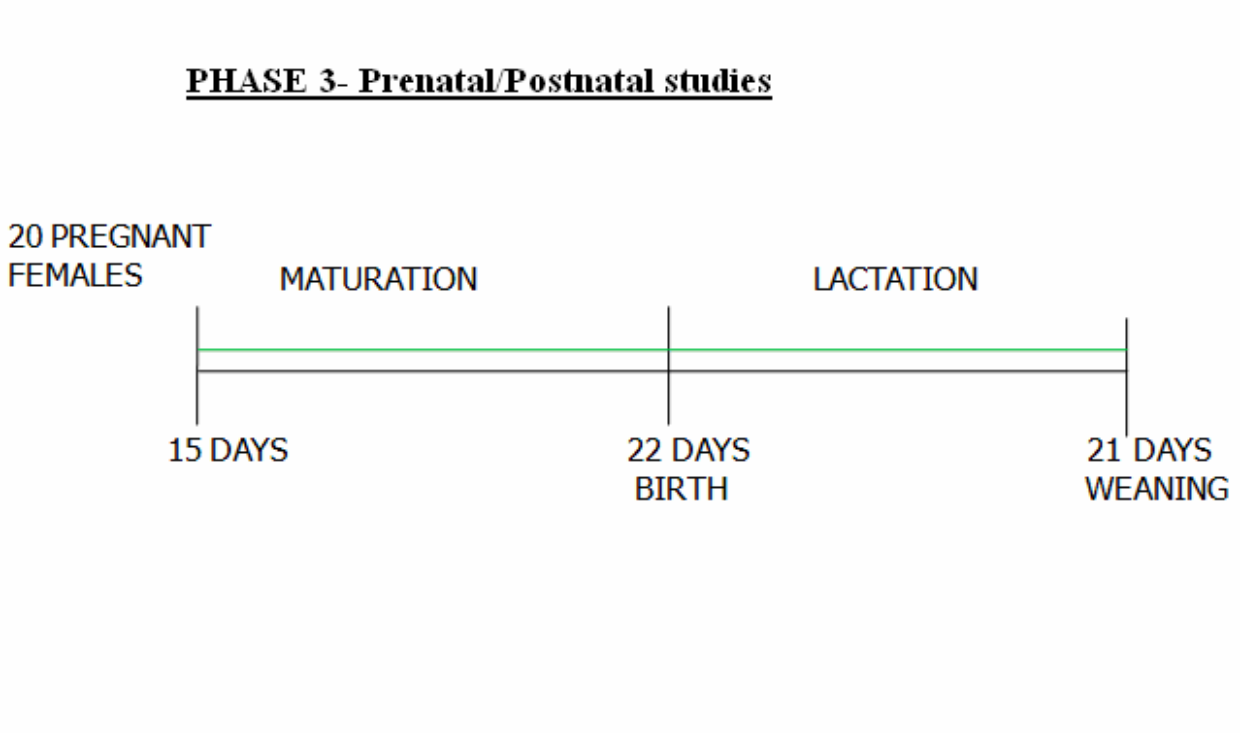
Teratological study-This is based on the principle that the embryo is most susceptible to induction of birth defects during the organogenesis period.



In this experiment 20 inseminated females mice per test group will be treated only during the organogenetic period.

One day prior to birth ,the dams are sacrificed and fetuses will be made to deliver by hysterectomy ; weighed, sexed and examined for gross, visceral and skeletal abnormalities.

Prenatal/Postnatal Study-The study will be done to determine the effects on late fetal development, labour and delivery ,lactation, neonatal viability and growth of the offspring.



In the given experiment, 20 inseminated females mice per test group will be treated during the last quarter of the pregnancy and through lactation to weaning period.

PARAMETERS:

Following parameters will be studied:

1. Number of live fetuses

2. Number of dead fetuses

3.Indices:

i) Viability Index: It will be calculated on days 4,7,14,21 of age by formulae:

viability index at 4 days of age = no. of alive pups on postnatal day 4/ no. of pups born alive

ii) Weaning Index : no. of pups alive at 21 days of age/no. of pups mentioned at 4 day of age

iii) Growth Index: Average weight of offspring at birth and on 4,7,14 and 21 days of age.

iv) Sex Ratio : No. of male and female offspring.

4. Behavioural tests

i) Pivoting: The pup moves in a circle using its front legs and head only, its hind legs do not move.

ii) Surface Righting: pups will be directly placed on their back on a flat table top and released. Time will be recorded until all four paws will return to the table surface.

iii) Tail hang reflex: It will be measured by holding the pup by its tail such the head is downwards. A positive response will be defined as flexing of the torso to raise the head.

3.Behavioural Parameters (2 minutes)

i) Locomotion: The total number of sectors entered by the animal.

ii) Rearing: The number of times the animal raise into its hind paws.

iii) Grooming: The number of times the animal touches its snout with the paws.

iv) Defecation: Number of times the animal defecates.

v) Exploratory behavior.

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